

A protocol for preparing subfossil chironomid head capsules (Diptera: Chironomidae) for stable isotope analysis in paleoclimate reconstruction and considerations of contamination sources

Y. Wang · D. R. Francis · D. M. O'Brien ·
M. J. Wooller

Received: 4 September 2007 / Accepted: 1 February 2008 / Published online: 20 February 2008
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Abstract Several techniques are available to examine the isotopic composition of historic lake waters, providing data that can subsequently be used to examine environmental changes. A recently-developed technique is the stable oxygen isotope analysis of subfossil chironomid (Diptera: Chironomidae) head capsules (mostly chitin) preserved in lake sediments. This technique involves a high Temperature Conversion Elemental Analyzer (TC/EA), which has been a relatively recent addition to the suite of online peripherals for analyzing the stable isotopic composition of organic samples. The highly precise and

accurate $^{18}\text{O}/^{16}\text{O}$ and D/H measurements obtainable using the TC/EA with samples in the microgram range make this instrumentation suitable for studying geochemical and biological processes. Preparation of organic samples for isotopic analysis typically requires first weighing each sample into silver/tin capsules. These capsules can introduce oxygen and hydrogen contamination (a “blank effect”), which is especially problematic for analysis of small organic samples (e.g. less than 100 μg). Here we tested tin and silver capsules from two manufacturers and a range of sizes to assess contamination to small organic samples on the TC/EA. We also assessed how a method for cleaning silver capsules affected our analysis of commercial chitin. In general, capsules made of silver have less detectible oxygen than those made of tin, and capsules from the two manufacturers varied in their detectible oxygen. There was no detectable H contamination from silver capsules. In addition to our empirical findings, we present a model demonstrating the influence that contaminant oxygen can have on the $\delta^{18}\text{O}$ of small organic samples. Sample mass becomes an important issue for such analyses. In light of our findings, we recommend a minimum sample mass $\geq 50 \mu\text{g}$ (approximately 120 whole chironomid head capsules) on a TC/EA-IRMS (Delta^{plus} XP system). Finally, we present a detailed protocol for preparing and transferring chironomid head capsules into silver capsules that minimizes the influence of contaminant oxygen. This protocol provides the paleo-community with another potential method for reconstructing paleoenvironments.

Y. Wang (✉) · M. J. Wooller
Alaska Stable Isotope Facility, Water & Environmental
Research Center, University of Alaska Fairbanks,
Fairbanks, AK 99775, USA
e-mail: ftyw@uaf.edu

Y. Wang
Department of Geology & Geophysics, University of
Alaska Fairbanks, Fairbanks, AK 99775, USA

D. R. Francis
Department of Geosciences, University of Massachusetts
Amherst, Amherst, MA 01003, USA

D. M. O'Brien
Institute of Arctic Biology, University of Alaska
Fairbanks, Fairbanks, AK 99775, USA

M. J. Wooller
School of Fisheries and Ocean Sciences, University of
Alaska Fairbanks, Fairbanks, AK 99775, USA

Keywords TC/EA-IRMS · Blank silver capsule · Tin capsule · Chitin · Stable oxygen and hydrogen isotopic compositions · Chironomidae

Introduction

Analyses of the stable oxygen and hydrogen isotope composition of biogenic materials have been used to reconstruct the stable isotopic composition of past precipitation, which is subsequently used to infer past environmental changes. The most commonly used methods in limnological isotope studies are the $\delta^{18}\text{O}$ analyses of carbonates from benthic ostracods (Hu et al. 2003), *Chara* sp. calcites (Anderson et al. 2001), biogenic silica (Lamb et al. 2004; Lamb et al. 2005; Leng and Barker 2006) and aquatic cellulose (Anderson et al. 2001; Sauer et al. 2001; Wolfe et al. 2000; Wolfe et al. 2001; Wolfe et al. 2007). The δD of beetle chitin (Grocke et al. 2006), aquatic biomarkers (Huang et al. 2004) and total organic matter (Wooller et al., 2007) have also been used to examine the past isotopic composition of precipitation and paleoclimate change.

These analyses have been facilitated by the development of online continuous flow instruments for stable isotope analysis of organic and inorganic samples, in particular, the High Temperature Conversion Elemental Analyzer (TC/EA) (Farquhar et al. 1997; Kelly et al. 1998; Kornexl et al. 1999a; Koziel 1997; Werner 2003; Werner et al. 1996), which determines D/H and $^{18}\text{O}/^{16}\text{O}$ stable isotope ratios in a rapid, easy, and precise fashion (Koziel 1997; Sharp et al. 2001). This on-line practice has reduced the sample mass required for analysis by an order of magnitude (Sharp et al. 2001; Werner et al. 1996), allowing analysis of samples in the microgram range (Farquhar et al. 1997; Kornexl et al. 1999a) and has made the technique suitable for studying natural geochemical and biological processes.

$\delta^{18}\text{O}$ analyses of subfossil chironomid (Diptera: Chironomidae) head capsules in lake sediments via TC/EA have been proposed as a way of examining past environmental conditions (Wooller et al. 2004; Wooller et al. 2007). Despite the potential of this approach, an explicit description of a protocol for the preparation of chironomid head capsules (hereafter referred to as fossil heads) for stable isotope analyses (H and O) using the TC/EA is lacking from the

current literature. The paper by Wooller et al. (2004) described a modern calibration set and two examples of the method's application to paleo reconstruction, but did not present the steps involved in preparing the samples. Recent studies devoted to $\delta^{18}\text{O}$ analyses of biogenic materials (e.g. cellulose, diatoms and human hair) have emphasized the need for careful attention to the details associated with sample-handling protocols (Bowen et al. 2005; Leng and Barker 2006; Wassenaar and Hobson 2002; Wolfe et al. 2007). Here we contribute to this literature with a detailed assessment of methodological considerations for analyzing the $\delta^{18}\text{O}$ and δD of fossil chironomid heads via TC/EA.

Previous work with fossil chironomid heads required very large numbers (300–700) to obtain enough sample mass for analysis (Wooller et al. 2004), and was time consuming. Reducing the sample mass (fossil head numbers) can result in a decrease in the sample voltage signal to noise ratio during $\delta^{18}\text{O}$ analyses. This occurs if empty tin or silver capsules (vessels used to contain samples for analysis) are contaminated with oxygen and hydrogen. The result is an oxygen or hydrogen voltage signal from the empty, or blank, capsules (hereafter referred to as a 'blank' effect) (Farquhar et al. 1997; Werner 2003; Werner and Brand 2001; Werner et al. 1996). The influence of the blank effect is naturally much greater when small sample masses are analyzed. A 'blank' signature has the potential to significantly affect the accuracy and precision of analyses of small-sized organic samples.

Capsules used for TC/EA analysis of organic samples are available in both silver and tin, and come in a variety of sizes. One might hypothesize that capsule sizes may influence the blank differently, for instance, the larger capsules may carry a larger blank signature. The different capsule material (either tin or silver) could also influence the blank differently. However, analyses in the published literature using the TC/EA have been conducted using both tin and silver capsules (Farquhar et al. 1997; Kelly et al. 1998; Kornexl et al. 1999a; Kornexl et al. 1999b; Kreuzer-Martin et al. 2003), and a direct comparison of their blank effects has not been conducted.

Tin capsules are cheaper, leave fewer residues in the reactor furnace of the TC/EA (Alaska Stable Isotope Facility, personal communications), and don't react to acidic samples (detailed discussions

see ISOGEOCHEM item number: 014870). However, Thermo Finnigan INC. suggests using silver capsules (Thermo Finnigan operation manual, 2003) for isotopic analysis on the TC/EA connected to an Isotope Ratio Mass Spectrometer (IRMS), because the glassy carbon reactors can be easily cleaned after running silver capsules whereas the whitish-grey coat left behind after using tin capsules is not so easily removed (SIRFER laboratory, personal communication; ISOGEOCHEM Listserv archive item number: 014856). Different users weigh these advantages and disadvantages when deciding which type of capsule to use, and as yet there is no clear consensus. Here we investigate the methodological considerations behind the analysis of small organic samples for $\delta^{18}\text{O}$ (and δD) using continuous flow TC/EA isotope ratio mass spectrometry. We examined the blank effect associated with silver and tin capsules of a variety of sizes from multiple batches and two different manufacturers. We also tested whether a cleaning procedure will decrease the blank effect. In addition, since chironomid sorting and transferring involves distilled water as a medium, we also tested whether wetting silver/tin capsules influenced the blank signal. We also modeled the effect of blanks of varying size and isotope composition on sample analyses of $\delta^{18}\text{O}$, to provide guidelines for minimum sample sizes and blank tolerance. Finally, we provide a clear outline of a protocol for preparing chironomids for stable isotope analysis to promote this new proxy for studying environmental changes.

Materials and methods

Assessment of blank effects from empty capsules

We measured the blank effect associated with different sizes of silver and tin capsules from two manufacturers

(Manufacturer 1: silver: 5×3.5 mm, 6×4 mm, and 9×5 mm; tin: 5×3.5 mm and 6×4 mm; and Manufacturer 2: silver: 4×3.2 mm, 3.75×3.5 mm, and 6×4 mm; tin: 3.75×3.5 mm and 4.74×4 mm) (Table 1). The capsules are packaged in plastic jars by the manufacturers, and batch numbers are provided to denote the production at different times. Therefore, several jars and batches were chosen to examine the degree of variability on blank signatures. The capsules to be analyzed were folded into balls and stored in 96-position culture trays (Elisa plates) covered with a lid. The capsules were then loaded into a zero blank auto sampler attached to an on-line pyrolysis thermochemical reactor (ThermoElectron TC/EA) coupled via a ConFlo III with a thermoFinnigan Delta^{plus} XP IRMS at the Alaska Stable Isotope Facility (ASIF). In order to examine any wetting/drying effect on the empty silver capsules, we also rinsed a series of different sized capsules (silver and tin) from manufacturer 1 and all sizes of capsules except for size 6×4 mm silver and 4.75×4 mm tin capsules from manufacturer 2 with Type I ultrapure water (18.2 MW cm resistivity at 25°C and <10 ppb Total Organic Carbon). These capsules were left to thoroughly air dry for 24 h and were then analyzed as above. Blank signals were reported as voltage peaks generated by the oxygen and hydrogen mass from blank capsules on the Delta^{plus} XP IRMS. The minimum peak detection limit for the TC/EA used at ASIF is smaller than 50 mV (0.05 V).

Examination of a cleaning treatment

Some laboratories and manufacturers clean silver capsules before loading samples to reduce the blank effect. We examined the effect of applying a cleaning method for silver capsules on $\delta^{18}\text{O}$ and δD analyses of

Table 1 Average weights, materials, and sizes of capsules from two different manufacturers used in the study

Capsule materials	Manufacturer 1		Manufacturer 2	
	Size (mm × mm)	Weight (mg)	Size (mm × mm)	Weight (mg)
Silver	5×3.5	11.62 ± 0.66 ($n = 26$)	4×3.2	13.80 ± 0.33 ($n = 10$)
	6×4	18.81 ± 0.52 ($n = 10$)	3.75×3.5	13.72 ± 0.48 ($n = 10$)
	9×5	36.87 ± 1.38 ($n = 10$)	6×4	28.52 ± 0.61 ($n = 10$)
Tin	5×3.5	10.21 ± 0.12 ($n = 10$)	3.75×3.5	5.70 ± 0.06 ($n = 10$)
	6×4	15.29 ± 0.28 ($n = 10$)	4.75×4	8.19 ± 0.11 ($n = 10$)

a chitin standard. Silver capsules (size 5×3.5 mm) from manufacturer 1 were selected for examining the cleaning method because this type produced undetectable oxygen in initial tests.

Thirty 5×3.5 mm silver capsules were rinsed twice using 100% acetone and left to air-dry for 24 h. Thirty-seven untreated 5×3.5 mm silver capsules were used as a control. We used pure homogenous chitin (made of crab shell, Sigma Organic Co. 2002) as our sample material. Chitin was weighed into the capsules using a microbalance (M2P Sartorius, repeatability 0.001 mg). Sample mass varied between 0.016 and 0.427 mg. All of the capsules were then folded into a small ball and analyzed on the TC/EA connected with IRMS at the ASIF. Standards (NBS18, NBS19, NBS22, NBS30, ANU sucrose, IAEA-601 and IAEA-602), and an internal organic (keratin) laboratory standard (BWBI) were analyzed with each run of chitin samples (measured versus expected $r^2 > 0.98$). ASIF internal laboratory standard benzoic acids (Fisher Scientific, No. 947429) were analyzed after every ten samples as a precision check. Analytical precision for $\delta^{18}\text{O}$ and δD was 0.6 and 4‰, respectively. All chitin results of $\delta^{18}\text{O}$ and δD are reported in units of per mil (‰) relative to Vienna Standard Mean Ocean Water (V-SMOW): $\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000\text{‰}$, where X is oxygen, R is the ratio of heavy to light isotope, and the standard is Vienna Standard Mean Ocean Water (V-SMOW). A one-way ANOVA was performed to compare chitin $\delta^{18}\text{O}$ using the cleaning methods against the control. We also compared the variances of $\delta^{18}\text{O}$ from the cleaning treatment and the control using Brown-Forsythe's test. All analyses were performed using the statistical package SAS 8.0.

Modeled influence of blanks on stable oxygen isotope analyses

A two end-member mixing model (1) was used to illustrate the contribution of isotopic composition from sample and blank sources:

$$\delta^{18}\text{O}_M = \delta^{18}\text{O}_S * p_S + \delta^{18}\text{O}_B * p_B \quad (1)$$

$$1 = p_S + p_B \quad (2)$$

where M = Measured, S = Sample, B = capsule Blank effect, and p_S and p_B = the oxygen proportional contribution of sample and blank, respectively. This expression has 3 unknowns: p_S , $\delta^{18}\text{O}_S$, and

$\delta^{18}\text{O}_B$, and is the same for δD measurements. By including several blank capsules in the sample sequences, the $\delta^{18}\text{O}_B$ and the magnitude of the blank effect (in Volts) can be measured. The p_B can be calculated for each sample as $p_B = \text{Blank peak area} / \text{Total peak area}$. The isotopic composition of individual samples ($\delta^{18}\text{O}_S$) can therefore be corrected by rearranging the mixing model (1) and (2).

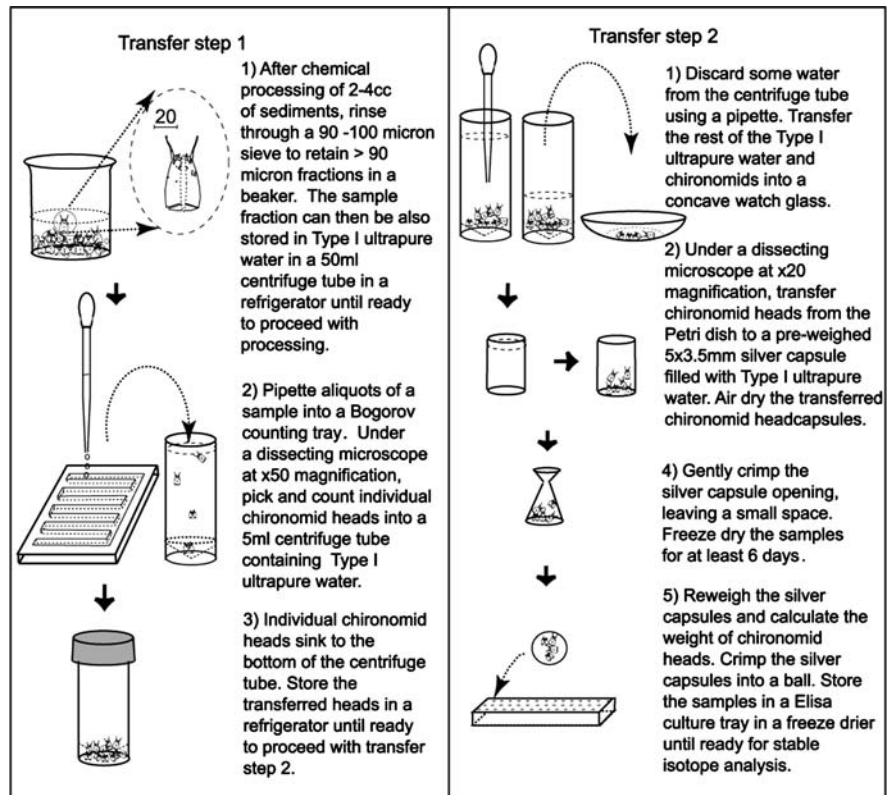
$$\delta^{18}\text{O}_S = \frac{\delta^{18}\text{O}_M - \delta^{18}\text{O}_B * p_B}{1 - p_B} \quad (3)$$

Correction for a blank effect is usually small when a large sample mass is used. When analyzing small-mass samples, however, the correction can be significant. Here we varied $\delta^{18}\text{O}_B$ and p_B to explore the effect of blanks of differing size and isotopic composition on $\delta^{18}\text{O}_M$. We used two model scenarios. In the first model (equation 1) we kept $\delta^{18}\text{O}_S$ and the blank oxygen proportional contribution constant (e.g. $p_B = 0.6$), and varied the isotope signature of the blank ($\delta^{18}\text{O}_B$) from -20 to 10 ‰ (these values are within the range we detected for $\delta^{18}\text{O}_B$ in our experiments with blanks at ASIF). We assigned the value of $\delta^{18}\text{O}_S$ to be 20.5‰ , the mean of the standard chitin measured in ASIF (see above). We then performed this simulation over a range of other possible blank oxygen percentage contributions ($p_B = 0, 0.15, \text{ and } 0.3$). These ranges of blank contributions were selected in the model simulation because we have experienced blank oxygen proportional contributions as high as 0.6 in prior analyses with small sample masses. In the second model we varied the percentages of the blank oxygen contribution but kept $\delta^{18}\text{O}_B$ (-7.7‰ , the mean blank $\delta^{18}\text{O}_S$ value from our experiments) and $\delta^{18}\text{O}_S$ (20.5‰ , as a mean value for chitin analyzed at ASIF) constant.

A protocol for preparing chironomids for stable isotope analysis

We investigated methods for cleaning and transferring chironomid fossil heads from sediment samples into silver capsules for stable isotope analysis. Here we present a description of the preparation protocol that we determined to be the most efficient (Fig. 1). About two to four cubic centimeters (cc) of lake core sediment is often sufficient to obtain enough chironomid fossil heads. Extraction of fossil heads from

Fig. 1 A schematic description showing the protocol for transferring chironomid fossil heads for stable isotope analysis



sediments follows the procedure described by Walker (2001). Samples are first treated with 10% HCl for 24 h, and then 5% KOH in a warm (60–70°C) water bath for 15–20 min. Between HCl and KOH treatment, the sediments are rinsed with distilled water in a 100 µm sieve (Walker 2001). After these processing steps, the residual material is refrigerated in a 50 ml centrifuge tube until it is ready for sorting.

The treated samples are sorted with two hand picking and transfer steps (Fig. 1). In the first transfer step, small aliquots of aqueous residual material are transferred using a pipette into a Bogorov counting tray (Gannon 1971) and examined under a dissecting microscope at 25–50× magnification. Fossil chironomid heads are transferred using fine forceps into 5 ml plastic centrifuge vials (VWR Scientific Product) containing Type I ultrapure water (hereafter, “water”). The vials are stored at 4°C until ready to proceed with the next transfer step. The second step involves transferring the fossil heads into a silver capsule for isotopic analysis. Excess water is removed from the centrifuge tube carefully so as not to disturb fossil heads that have settled to the base

of the tube from the first step. The remaining water and concentrated heads are then gently poured into a concave watch glass. We use a few extra drops of water to rinse the tube and ensure that no chironomid fossil heads remain stuck to the inner wall of the centrifuge tube. Using fine forceps or a fine tip paint brush and a dissection scope at 50× magnification, a minimum of 120 chironomid heads are transferred into pre-weighed silver capsules filled with water. If water evaporates during the process, additional water sometimes is added to refill the capsules. In order to prevent the silver capsules from tipping over, an inexpensive holder can be made from an appropriately sized nut from a nut-and-bolt assembly. When all the heads are transferred into the silver capsules, the open silver capsules are allowed to air dry over night. The capsules are then crimped carefully to leave a small opening and freeze dried for ≥6 days (Bowen et al. 2005). The silver capsules are then reweighed and the mass of each sample is calculated by subtracting the tare weight. Silver capsules are then folded into a tiny silver ball and stored in an Elisa culture tray in a freeze drier or a vacuum system

prior to loading them into a zero blank auto-sampler attached to the TC/EA-IRMS described above for the stable isotope analysis. Benzoic acid and blank silver capsules are placed into the auto-sampler along with other international standards as described in the chitin experiments above.

It is worth noting that during sedimentation processes and chemical treatments, some mouthparts may be lost or detached or whole heads can break into two pieces. We experienced that many fossil heads tend to break along the middle line, so every two half heads were counted as one.

Results

Assessment of blank effects from empty capsules

The blank effects (in Volts) from silver and tin capsules over a range of sizes and from two manufacturers are presented in Table 2. All sizes (5 × 3.5 mm, 6 × 4 mm, and 9 × 5 mm) of silver capsules from manufacturer 1 showed no detectable

oxygen blank signal (Table 2 and Fig. 2), either unrinsed or rinsed. Only size 9 × 5 mm silver capsules produced a detectable hydrogen blank signal, and did so for both rinsed and unrinsed capsules (mean = 0.02 (±0.01) Volts). Both sizes of tin capsules from manufacturer 1 had detectable oxygen blank voltages, and a blank hydrogen voltage was observed in the 6 × 4 mm tin capsule only. Rinsing appeared to cause all blank voltages, when present, to increase slightly. Although production batch numbers were not supplied by manufacturer 1, the 5 × 3.5 mm silver capsules were ordered in both 2003 and 2004, and both produced undetectable blanks. One Volt of oxygen is generated by ~30 µg oxygen sample mass on the Delta^{plus} XP TC/EA-IRMS system at ASIF. One Volt of hydrogen is generated by ~5.5 µg hydrogen sample mass.

All unrinsed and rinsed silver and tin capsules from manufacturer 2 produced detectable oxygen blank signals, but no detectable hydrogen blank signals (Table 2 and Fig. 2). Rinsing appeared to decrease the blanks slightly. However, there were no significant differences in oxygen signals from rinsed

Table 2 Results from empirical tests of capsule types from two different manufacturers

Manufacturer	Capsule materials	Sizes (mm × mm)	Treatment	Detected blank oxygen voltages	Detected blank hydrogen voltages
1	Silver	5 × 3.5	Unrinsed	0 (<i>n</i> = 16)	0 (<i>n</i> = 16)
			Ultrapure water rinsed	0 (<i>n</i> = 16)	0 (<i>n</i> = 16)
		6 × 4	Unrinsed	0 (<i>n</i> = 10)	0 (<i>n</i> = 10)
			Ultrapure water rinsed	0 (<i>n</i> = 10)	0 (<i>n</i> = 10)
		9 × 5	Unrinsed	0 (<i>n</i> = 10)	0.02 ± 0.01 (<i>n</i> = 10)
			Ultrapure water rinsed	0 (<i>n</i> = 10)	0.02 ± 0.01 (<i>n</i> = 10)
	Tin	5 × 3.5	Unrinsed	0.08 ± 0.02 (<i>n</i> = 20)	0 (<i>n</i> = 20)
			Ultrapure water rinsed	0.09 ± 0.02 (<i>n</i> = 20)	0.01 ± 0.03 (<i>n</i> = 20)
		6 × 4	Unrinsed	0.12 ± 0.03 (<i>n</i> = 20)	0.08 ± 0.01 (<i>n</i> = 20)
			Ultrapure water rinsed	0.14 ± 0.05 (<i>n</i> = 20)	0.12 ± 0.09 (<i>n</i> = 20)
2	Silver	4 × 3.2	Unrinsed	1.00 ± 0.03 (<i>n</i> = 14)	0 (<i>n</i> = 14)
			Ultrapure water rinsed	0.68 ± 0.49 (<i>n</i> = 10)	0 (<i>n</i> = 10)
		3.75 × 3.5	Unrinsed	0.91 ± 0.34 (<i>n</i> = 18)	0 (<i>n</i> = 18)
			Ultrapure water rinsed	0.50 ± 0.64 (<i>n</i> = 10)	0.02 ± 0.07 (<i>n</i> = 10)
		6 × 4	Unrinsed	1.70 ± 0.45 (<i>n</i> = 15)	0 (<i>n</i> = 15)
			Ultrapure water rinsed	Not tested	Not tested
	Tin	3.75 × 3.5	Unrinsed	0.92 ± 0.28 (<i>n</i> = 18)	0 (<i>n</i> = 18)
			Ultrapure water rinsed	0.66 ± 0.37 (<i>n</i> = 10)	0 (<i>n</i> = 10)
		4.75 × 4	Unrinsed	1.18 ± 0.35 (<i>n</i> = 20)	0 (<i>n</i> = 20)
			Ultrapure water rinsed	Not tested	Not tested

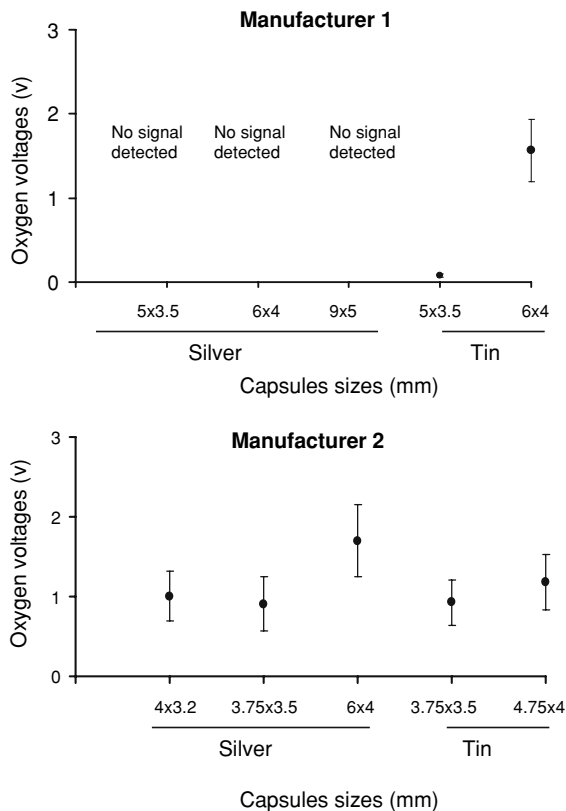


Fig. 2 Blank signals (Volts) detected from capsules of various sizes and materials from manufacturers 1 and 2

and unrinsed capsules (all $P > 0.06$ for each size). The three sizes of silver capsules ($n = 55$) showed a significant difference in their oxygen blank ($P < 0.0001$, ANOVA). The smaller the size of silver capsules from manufacturer 2, the less the oxygen blank effect. Oxygen blanks from two sizes of tin capsules ($n = 32$) did not show significant differences ($P = 0.37$). We compared two production batches of each capsule size from manufacturer 2, and found no significant differences in the amount (voltage) of blank (t -test, all $P \geq 0.14$) except for 3.75×3.5 mm silver capsules ($P = 0.04$).

The detectable blank $\delta^{18}\text{O}$ produced from capsules ($n = 34$) ranged from -31 to 10 ‰ with a mean of $-7.7 (\pm 10.9)$ ‰. The repeatability of these blanks was low.

Examination of a cleaning treatment

The chitin contained in silver capsules ($n = 42$) that had been rinsed in acetone yielded a mean $\delta^{18}\text{O}$ of

Table 3 Chitin $\delta^{18}\text{O}$ versus for samples prepared in 5×3.5 mm silver capsules rinsed in acetone and untreated 5×3.5 mm silver capsules

	Treatment 1 ^a	Untreated
Mean $\delta^{18}\text{O}$ (all samples)	22.2 ± 1.3 ($n = 42$)	20.6 ± 0.7 ($n = 37$)
Mean $\delta^{18}\text{O}$ (voltages >0.5 V)	21.9 ± 1.2 ($n = 36$)	20.5 ± 0.2 ($n = 33$)

^a Treatment 1 = rinsed in acetone

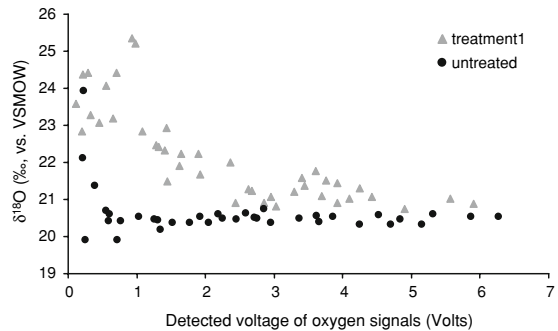


Fig. 3 Chitin $\delta^{18}\text{O}$ versus the detected voltages of oxygen signals for samples prepared in untreated 5×3.5 mm silver capsules and 5×3.5 mm silver capsules rinsed in acetone

22.2% (± 1.3), whereas the chitin from untreated silver capsules ($n = 37$) yielded a mean of 20.6% (± 0.7) (Table 3 and Fig. 3). The variance in the chitin $\delta^{18}\text{O}$ from the untreated capsules was significantly lower than acetone rinsed capsules ($P < 0.0001$, Brown-Forsythe variance test). Measurements of chitin $\delta^{18}\text{O}$ were highly consistent from untreated capsules, with the exception of samples having voltages below 0.39 V (Fig. 2). For small sample masses (<0.50 V ≈ 50 μg sample) the signal to noise ratio becomes large. If we remove these small voltages (<0.50 V), the mean value of pure chitin in untreated silver capsules ($n = 33$) was 20.5% (± 0.2) (reported here as the true value of chitin from the untreated silver capsules), whereas acetone rinsed silver capsules ($n = 36$) yielded a mean $\delta^{18}\text{O}$ of 21.9% (± 1.2).

Modeled influence of blanks on stable oxygen isotope analyses

The $\delta^{18}\text{O}$ values for small organic samples are composed of two end-members: the organic sample

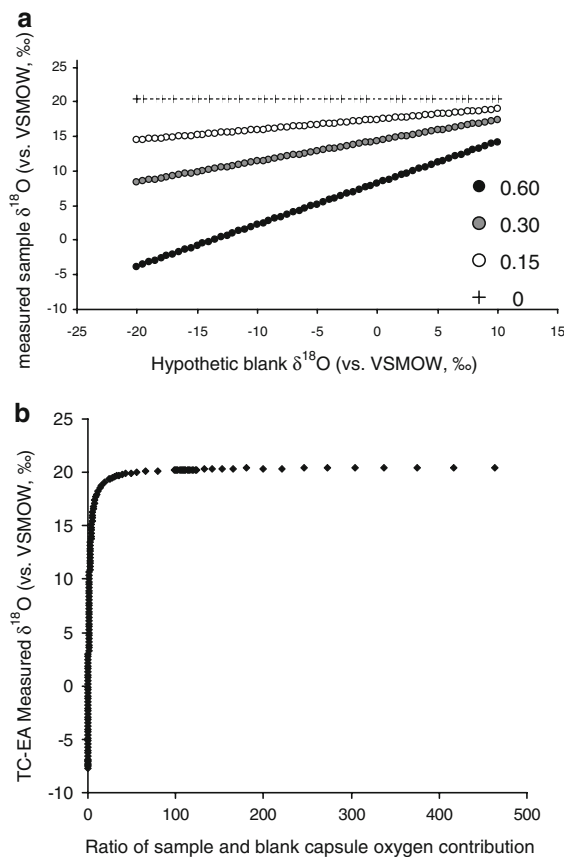


Fig. 4 (a) A series of mixing models demonstrating the influence of different quantities of blank oxygen on $\delta^{18}\text{O}$ of a sample. By keeping sample $\delta^{18}\text{O}$ constant (20.5‰) and varying the $\delta^{18}\text{O}$ of the blank capsules (−20 to 10‰), the measured sample values vary depending on the oxygen contribution (0, 0.15, 0.30 and 0.60 respectively) of the blank capsule oxygen contribution. The higher the proportion of blank oxygen the larger the difference a sample will be from its true value, (b) A mixing model of blank oxygen and sample oxygen. The model involves keeping the $\delta^{18}\text{O}$ of blank oxygen (−7.7‰) and sample oxygen (20.5‰) constant and varying the proportional contributions of the two end members to the total oxygen concentration. The measured values converge on the real sample $\delta^{18}\text{O}$ value as the sample oxygen becomes much greater than blank oxygen

oxygen and the capsule contaminant or blank oxygen. Our model assumes that $\delta^{18}\text{O}_\text{S}$ (20.5‰) and blank oxygen contribution are constant as 0.60, but $\delta^{18}\text{O}_\text{B}$ varies from −20 to 10‰ (Fig. 4a). The measured sample $\delta^{18}\text{O}_\text{M}$ is positively correlated with the $\delta^{18}\text{O}_\text{B}$ and the $\delta^{18}\text{O}_\text{M}$ have a wide range of values. We repeated the model by assigning different blank oxygen contributions (e.g. 0, 0.15, and 0.30). The smaller the oxygen percentage from the blank

capsules, the closer the measured $\delta^{18}\text{O}_\text{M}$ to the corrected $\delta^{18}\text{O}$ (true sample value). In other words, the slope of correction decreases as the percentage of blank oxygen contribution decreases. When the blank oxygen from the capsules is 0%, the measured $\delta^{18}\text{O}$ is the true sample value. In model scenario 2 we kept the $\delta^{18}\text{O}_\text{B}$ (−7.7‰, the mean of $\delta^{18}\text{O}_\text{B}$) and $\delta^{18}\text{O}_\text{S}$ (20.5‰) constant, and varied the ratio of sample and blank capsule oxygen contribution. The result (Fig. 4 b) showed that when sample oxygen percentage is much greater than the blank oxygen percentage (the ratio of sample and blank capsule oxygen contribution is large), the measured value $\delta^{18}\text{O}_\text{M}$ is closer to the true sample value $\delta^{18}\text{O}_\text{S}$.

A protocol for preparing chironomids for stable isotope analysis

Using silver capsules with no measurable blank allowed us to decrease the number of chironomid fossil heads from 300–700 to approximately 120 (>50 μg) with a variety of sizes, and still produce an adequate signal (at least 0.5 V on the Delta^{plus} XP TC/EA-IRMS at ASIF). This new approach has been used to analyze stable isotopes (C, N and O) on chironomid fossil heads from a lake core from Northern Iceland (Wooller et al. 2007). Triplicate analyses of chironomid fossil heads (>50 μg) from the same depth from a core of lake sediment from an Icelandic lake produced a standard deviation of 0.6‰ (Wooller et al. 2007).

Discussion

Our analyses of the blank effects from empty capsules showed that blank oxygen and hydrogen voltages were dependent on the manufacturer, materials (silver and tin), and capsule size. Type I ultrapure water rinsing did not affect the oxygen blank signals significantly on any types and sizes of capsules, which ensured that using water as a medium during chironomid transferring and picking has no detectable influence on the blank effect. Our numerical model showed that the $\delta^{18}\text{O}$ and percentage of the blank signal from tin or silver capsules can have a significant influence on the $\delta^{18}\text{O}$ of small organic samples. Silver capsules from manufacturer 1 had the lowest detectable oxygen voltages compared to tin

capsules. This finding is consistent with the discussions on ISOGEOCHEM (item number: 014854). Tin capsules of different sizes (5×3.5 mm and 6×4 mm) from manufacturer 1 showed significant differences in both oxygen and hydrogen blank voltages. The larger the capsule size, the larger the blank signals.

Significant differences in oxygen blank signals between different sizes of silver capsules were shown from manufacturer 2, which may suggest capsule size needs to be taken into consideration when sample sizes are limited. A majority of batches in the same size capsules have no significant differences of blank, although we observed that significant batch differences (3.75×3.5 mm silver capsules) of blank effect do occur. We therefore recommend restricting the capsules used in a particular study to one particular batch. We did not see significant differences in the two sizes of tin capsules from manufacturer 2. However, even though there were blank signals detected on the instrument, they were still very small in terms of their voltages compared to the voltage generated by a larger sample ($>1,000$ μg). Thus we recommend use of silver capsules when sample sizes are limited. We also recommend correcting for the blank effect by analyzing a series of blank silver or tin capsules used in sample preparation. Because the blank effect varies between manufacturers, we recommend that each stable isotope laboratory assess several manufacturers for different capsule sizes and materials before analyzing samples. A blank correction procedure comes with TC/EA operating software. However, manually conducted blank correction may be more appropriate because of the uncertainty of blank $\delta^{18}\text{O}$ and capsule size in use.

We have also noticed that silver capsules oxidized when stored containing benzoic acid for a considerable time. Oxidizations have been also observed when silver capsules are used to contain other acidic materials such as tomato juice (see discussion in Listserv in ISOGEOCHEM: Item No. 014857). Therefore, when handling acidic samples, we recommend avoiding silver capsules. We also recommend weighing benzoic acid standards just prior to analysis to avoid oxidization of silver capsules.

Although methods of cleaning silver capsules have been recommended by other laboratories, we found that the cleaning methods we investigated was not

necessarily effective at removing the contaminants from the silver capsules. We found that pure chitin in silver capsules that had been cleaned via acetone rinse yielded data with greater variance compared to those samples prepared in untreated silver capsules from the same manufacturer. The precision was markedly better for untreated capsules. We have also found (data not shown here) that chitin samples weighed into 5×3.5 mm silver capsules that had been baked in a high temperature furnace (400°C , purged with N_2 gas) for 24 h also showed large variance (Wang, unpublished data).

Our results showed the minimum chitin size to be ~ 50 μg (0.5 V) for analysis on our Delta^{plus} XP system TC/EA-IRMS, however, we recommend even larger sample masses (to at least generate 1 V) to further improve precision on this system (Fig. 3). The relatively newer Delta V IRMS also has much higher sensitivity compared to a Delta^{plus} XP. In this case, fewer chironomid heads would be required in order to yield a detectable signal. A test of the minimum oxygen sensitivity is required at the individual laboratory when considering measurement of relatively small samples mass.

Stable isotope analysis of chironomid fossil head capsules is a relatively new technique and only a few published applications have thus far been completed using this proxy. Compared to the first report using this technique (Wooller et al. 2004), this new study has significantly decreased the numbers of chironomid fossil heads (sample mass) needed for $\delta^{18}\text{O}$ analysis, which makes this approach less time consuming. We have divided the transferring procedure of chironomid fossil heads for isotope analysis into two steps, compared to Wooller et al. (2004), where the chironomid fossil heads were directly picked from a Bogorov counting tray into a silver capsule. One of the disadvantages of a one step transfer is that the sorting time is long, thus the first sample of one batch can end up stored in silver capsules for an extensive period of time compared to the last sample prepared. In the new protocol, an entire batch of samples can be sorted and stored in ultrapure water before proceeding with the second transfer step. This ensures that all samples in one batch are stored in the silver capsules for about the same length of time. The second transfer step also allows removal of other accidentally picked materials or misidentified parts from the sample. Chironomid

fossil heads picked and transferred in this manner can also be used as standard protocol for stable isotope analyses of other elements such as carbon, nitrogen (Wooller et al. 2007) and hydrogen. The smaller sample mass and two-step transferring protocol have resulted in much faster sample processing. However, even though we recommend a minimum of ~ 120 chironomid fossil heads (equivalent to 0.5 V of oxygen signal on a Delta^{plus} XP system at ASIF) the number should be based on the minimum (or greater) sample mass and the sensitivity of the instrument that is used. Chironomid heads also range in size due to their different instar stages, or different species. We have experienced some samples in which 50 large fourth instar larvae heads in an Icelandic lake core generated 50 μg (equivalent of ~ 0.5 V) and produced repeatable data (Wooller et al. 2007). Therefore the procedure for chironomid picking can be shortened if large instar larvae are preserved in the sediments. It is the sample mass that is the essential factor for organic isotopic analysis. We also suggest that other researchers assess the relative abundances of chironomid fossil heads from several depths before dedicating time to downcore $\delta^{18}\text{O}$ analyses.

Additional recommendation and future direction

Even though $\delta^{18}\text{O}$ of chironomids has been applied as a new proxy for past environmental changes, the complex origin of oxygen in chitin is not fully understood, since both water and diet can influence the $\delta^{18}\text{O}$ of chitin (Miller 1991; Miller et al. 1993; Schimmelmann and DeNiro 1986a). The degree to which water, diet, and physiology (e.g. temperature dependent fractionation) influence $\delta^{18}\text{O}$ in chitin is largely unexplored. The fundamental mechanisms controlling $\delta^{18}\text{O}$ in chironomids still need to be quantitatively understood. Therefore experimental, laboratory-based validations of isotope effects under a variety of conditions could provide an excellent model for simulating different environmental parameters to examine their influence on stable isotope composition (Gannes et al. 1997). Understanding the influence of water and diet on tissue $\delta^{18}\text{O}$ and the magnitude of temperature dependent physiological isotope fractionation will indeed lay the groundwork for a variety of paleoecological applications using $\delta^{18}\text{O}$.

Acknowledgements This research was supported by the National Science Foundation (NSF ESH-0317766 to Wooller) and the Alaska Stable Isotope Facility (ASIF) at the University of Alaska Fairbanks. The authors thank ASIF colleagues Tim Howe and Norma Haubenstock for their diligent work and valuable comments for experimental design and suggestions. We are grateful to participants and organizers, especially Dr. Ian Walker and Dr. Pete Landgon, at the international “Dead midges 2005 workshop” for stimulating encouragement for stable isotope applications on chironomid fossil heads. Thanks also to Dr. Yarrow Axford and Dr. Gifford Miller from the Institute for Arctic and Alpine Research (INSTAAR), Boulder, Colorado for valuable comments and support for method development in the early stages. We also thank Lena Krutikov and two anonymous reviewers for their constructive reviews of our paper.

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